

# The Antimetastatic Effect of Macrophages Restored by Indomethacin: Concomitant Tumor Immunity Model

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## ABSTRACT

*The role of macrophages acting as immunologic antitumor effectors and promoters of tumor growth are poorly understood as yet. We investigated the role of macrophage in model of concomitant immunity (CI), a phenomenon of secondary tumor rejection during the primary tumor growth. It has been shown that the period of CI weakening can coincide with appearance of tumor metastases. We used mammary carcinoma (MC) artificial lung metastases to evaluate the influence of macrophages from various period of CI on the development of metastases in mice. Our results indicated that macrophages are responsible for the late period of CI weakening and suppression. To investigate whether prostaglandins can mediate suppressive effect of macrophages we used experiments with indomethacin and we found that inhibition of prostaglandin E<sub>2</sub> synthesis by indomethacin restored antimetastatic effect of concomitant immune macrophages.*

**Key words:** macrophages, concomitant immunity, mammary carcinoma, prostaglandins

## Introduction

The role of macrophages in tumour disease and its interactions with other cell types, cytokines and diverse growth factors are poorly understood as yet. The most obscure is controversial role of macrophages acting as immunologic antitumor effectors and promoters of tumor growth. Thus, in some tumors and at the some steps of progression, macrophages may promote neoplastic proliferation, either directly by producing growth factors or indirectly, for instance, via the induction of immunosuppression or tumor angiogenesis<sup>1,2</sup>. On the other hand, the antitumor effect of macrophages is unspecific and rather powerful in elimination of tumour cells with poor expression of the MHC molecules or tumour antigens<sup>3</sup>.

The numerous studies showed direct casual relationship between tumour presence and dysfunction of various immunological antitumor mechanisms<sup>4,5</sup>. It has been demonstrated that macrophages contribute up to or more than half of tumor's mass<sup>6</sup>. Also, the more aggressive tumors, e.g. colon carcinoma Duke's C, exhibit pro-

found macrophage infiltration than the same type of tumor in earlier stadium<sup>7</sup>. It has been shown, that tumor cells recruit monocytes by production of chemotactic molecules, such as macrophage chemotactic proteins MCP-1, MCP-2, MCP-3, M-CSF, GM-CSF and IL-12p40 fragment<sup>8,9</sup>. Only after activation, the accumulated macrophages in the tumor tissue would exhibit antitumor cytotoxicity. There are two cytokines, IFN- $\gamma$  and TNF- $\alpha$ , which have the strongest effect on macrophages activation. Some other cytokines, e.g. GM-CSF, present in tumour tissue, could act as chemotactic molecule as well as activators of macrophages<sup>10</sup>.

Moreover, it has been proved in the various experimental and clinical studies that adoptive transfer of macrophages could elicit the antitumor immune response<sup>11</sup>. However, experimental trials using macrophages have produced different results ranging from effective antitumor response to inefficacy or even better tumour growth. These observations could be explained

by different level of activation of macrophages or rather various stages of differentiation, because macrophages could release and express various mediators which act antitumorocidal or tumor promoting<sup>12</sup>.

Macrophages stimulate tumor growth by secretion of immunosuppressive mediators or factors that stimulate angiogenesis – e.g. prostaglandin E<sub>2</sub> (PG E<sub>2</sub>) that is thought to have an important regulatory role in immune responses<sup>13</sup>. PG E<sub>2</sub> could be involved in regulation of the early stages of lymphocyte or macrophage activation<sup>14</sup>.

Our previous results indicated that macrophages could be important in late period of weakening of concomitant immunity (CI) to tumor<sup>15</sup>. Concomitant immunity is a phenomenon of secondary tumor rejection during the primary tumor growth which was described in many tumor models. It can be induced by both immunogenic and non-immunogenic factors, but its mechanism and dynamic differ, depending on tumor type and experimental model<sup>16</sup>. It appears after different time periods (depending on tumor type and experimental model), attains its peak and then subsides<sup>16</sup>. The period of CI weakening can coincide with appearance of tumor metastases<sup>17</sup>. Therefore, the investigation of mechanisms involved in CI weakening could contribute to better understanding of metastasing process. To resolve whether prostaglandin E<sub>2</sub> is involved in macrophage role in period of weakening of concomitant immunity we used indomethacin that is cyclooxygenase II inhibitor and therefore inhibits the synthesis of PG E<sub>2</sub>.

## Materials and Methods

### Animals

The experiments were carried out in CBA of both sexes, aged 3 to 4 months and weighting 18 to 24 g. Animals were maintained in acclimatized cages on a 12h light- dark cycle and had free access to standard laboratory chow and water. All experiments were conducted at Department of Physiology, School of Medicine, Zagreb University, Croatia and were approved by the local ethical committee.

### Tumor

Mammary carcinoma (MC) is maintained in our Department by regular subcutaneous passages. CBA mice that were previously subcutaneously transplanted with mammary carcinoma, were anesthetized with ketamine/xylazine to perform excision of tumor. Solid MC, after elimination of necrotic and fibrous tissues, was finely minced and placed in enzyme solution containing 1000 ml RPMI-1640 medium, 10 ml Hepes sodium salt solution, 0.1 g DNase, 2500 U of hyaluronidase and 1 g of collagenase (Sigma Aldrich). Twelve ml of solution were added to 2 g of tumor tissue, centrifugated (2000 g/10 min) at room temperature and washed three times in PBS. Tumor cells viability, determined by tripan blue, was above 95%.

### Spleen cell suspension

Mice with different exposure time to subcutaneous mammary carcinoma were spleen donors. The isolated spleens were cut in small pieces using scissors, passed through a stainless-steel mesh, centrifugated (2400 g/5 min) and washed in PBS three times. Tumor cells viability, determined by tripan blue under optical microscope, was above 95%.

### Isolation of macrophages from spleen cell suspension

Spleen cell suspension ( $10 \times 10^6$ ) were resuspended in RPMI-160 medium supplemented with 10% of fetal bovine serum and incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 120 minutes in a plastic petri dish. After the incubation, petri dishes were washed out twice with PBS. Adherent spleen cells were collected from the dish bottom by policeman and resuspended in RPMI-1640. The presence of macrophages in supernatant, tested by Indian ink phagocytosis assay, was above 95%. The number of viable cells was determined by tripan blue exclusion under optical microscope and always exceeded 90%.

### Adoptive transfer injection

For adoptive transfer injection we used spleenocytes suspension or macrophages alone from spleen of a subcutaneous mammary carcinoma host from different stages of concomitant immunity. The number of intravenous injected spleenocytes was equivalent to the number of cells in one spleen (spleen equivalent =  $80 \times 10^6$  spleenocytes in 0.5 mL of suspension). The number of adoptive transferred macrophages was  $8 \times 10^6$  cells, while the number of spleenocytes depleted with macrophages was  $80 \times 10^6$  cells. Animals were intravenous injected with  $1 \times 10^6$  MC cells one day after adoptive spleenocytes transfer.

### Indometacine therapy

Indometacine (kindly gift from Belupo) powder was dissolved in 95% ethanol until the concentration of 5 mg/ml and then diluted in PBS until the concentration of 200 µmol/l. Mice were intraperitoneally injected with 0.2 ml of indometacine solution 3 times in 2 weeks before the adoptive transfers of cells.

### Pulmonary metastases-counting assay

Animals were sacrificed 2 weeks following intravenous injection of  $5 \times 10^4$  MC cells. The trachea was isolated by midline chest incision and transected well above carina. Two ml of Indian ink solution (15% ink and 85% distilled water, with two drops of ammonia water added) were gently injected into the lung. The lungs were dissected *en block* from thoracic cage and placed in petri dish containing water for next 5 minutes. The pulmonary lobi were separated and placed in Fekete' solution (100 ml 70% ethanol, 10% formaldehyde and 5 mL of glacial acetic acid). The lung metastases were counted under lens control<sup>18</sup>.

### Statistical analysis

Distribution of data was tested with Kolmogorov-Smirnov test. Results were tested using analysis of variance.

### Results

To evaluate the antimetastatic effect of spleen cells from various periods of concomitant tumor immunity we designed the experiments as follows. In first set of experiment splenocytes from animals carrying tumor for 9, 16, 30 and 40 days were adoptively transferred intravenously. A day after each animal was i.v. injected by the suspension of tumor cells. The animals in control group received only tumor cells i.v. Two weeks later the number of metastases was counted. The results in Figure 1 show no difference between lung metastases number in animals that were adoptively transferred with normal splenocytes and control. The best antimetastatic effect was observed in animals adoptively transferred with splenocytes from mice carrying s.c. MC for 9 days, while the splenocytes from mice carrying s.c. MC for 16 days still exhibited antimetastatic effect, but weaker. The splenocytes from mice carrying MC for 30 or 40 days did not manifest antimetastatic effect as compared to groups mentioned above (Figure 1).

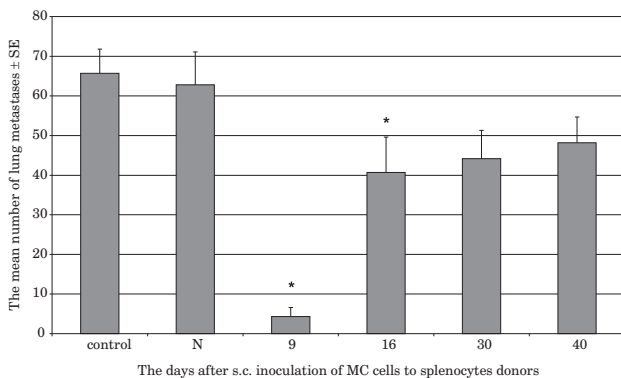


Fig. 1. The antimetastatic effect of adoptively transferred spleen cells from various periods of concomitant tumor immunity. Animals in control group received only  $1 \times 10^6$  MC cells i.v. N refers to splenocytes from non-tumor bearing mice. Adoptive transfer was performed by i.v. injection  $80 \times 10^6$  spleen cells following by the i.v. injection of  $1 \times 10^6$  MC cells.

\* $p \leq 0.05$  versus control

To explain the cellular mechanism involved in the weakening of antimetastatic effect of splenocytes from donors carrying tumors for 16, 30 and 40 days, we added those cells to concomitant immune splenocytes (splenocytes from mice carrying tumor for 9 days, spl<sub>9</sub>). That suspension of cells was adoptively transferred to mice followed by the i.v. injection of tumor cells. The results are shown in Table 1. In agreement with previously shown results, adoptively transferred concomitant immune splenocytes (Group 3) reduced lung metastases number, while adoptively transferred normal splenocytes (Group

TABLE 1  
THE ANTIMETASTATIC EFFECT OF ADOPTIVELY TRANSFERRED SPLEEN CELLS (SPL) FROM VARIOUS PERIODS OF CI ADDED TO CONCOMITANT IMMUNE SPLENOCYTES (SPL<sub>9</sub>)

Group	Adoptively transferred spleen cells*	The mean number of lung metastases ± SE
1	—	47.1 ± 7.3
2	spl <sub>N</sub>	32.2 ± 4.4 <sup>#</sup>
3	spl <sub>9</sub>	6.2 ± 1.7
4	spl <sub>9</sub> + spl <sub>N</sub>	7.7 ± 2.7
5	spl <sub>9</sub> + spl <sub>16</sub>	11.0 ± 2.1
6	spl <sub>9</sub> + spl <sub>30</sub>	36.4 ± 1.7 <sup>#</sup>
7	spl <sub>9</sub> + spl <sub>40</sub>	31.8 ± 4.9 <sup>#</sup>

\*Animals in group 1 received only  $1 \times 10^6$  MC cells i.v. Indices: 0, 9, 16, 30 and 40 refers to splenocytes from mice carrying MC subcutaneously for 9, 16, 30 or 40 days. N refers to splenocytes from non-tumor bearing mice. Adoptive transfer was performed by i.v. injection  $80 \times 10^6$  spleen cells following by the iv injection of  $1 \times 10^6$  MC cells.

<sup>#</sup> $p \leq 0.001$  versus group 3

2) showed no effect. Addition of splenocytes from mice carrying subcutaneous tumor for 30 or 40 days abolished antimetastatic effect of concomitant immune splenocytes. Those results indicate that in spleen cell suspensions from the period of CI weakening exists certain cells involved in immunosuppression. Although the spleen cells from mice carrying the tumor for 16 days lose their antimetastatic effect, when added to spl<sub>9</sub> did not suppress concomitant immune splenocytes. That phenomenon could be explained by the dynamic of CI where the 16 day could be considered as transient period toward CI weakening.

To evaluate the antimetastatic effect of macrophages from various periods of concomitant tumor immunity, we adoptively transferred i.v. macrophages from animals carrying tumor for 9, 16, 30 and 40 days. A day after each animal was i.v. injected by the suspension of tumor cells. The mean number of lung metastases is shown in Figure 2. The strongest antimetastatic effect had macrophages from mice carrying tumor for 16 days, while the macrophages from later CI periods became less effective. Macrophages from the later CI period (mice carrying s.c. MC for 40 days) not only lose their antimetastatic effect, but also started to promote the tumor growth (Figure 2).

We presumed that in spleen cell suspensions from the period of CI weakening (mice carrying s.c. MC for 30 and/or 40 days) exists certain cells involved in immunosuppression, we test if those cells could be macrophages. For that purpose we added macrophages from the donors carrying tumor for 9, 16, 30 and 40 days to concomitant immune cells and that suspension was adoptively transferred a day before i.v. injection of tumor cells. The results are shown in Table 2. The macrophages from mice carrying MC s.c for 16 days increased the antimetastatic effect of spl<sub>9</sub>. On the other hand, the macrophages from ani-

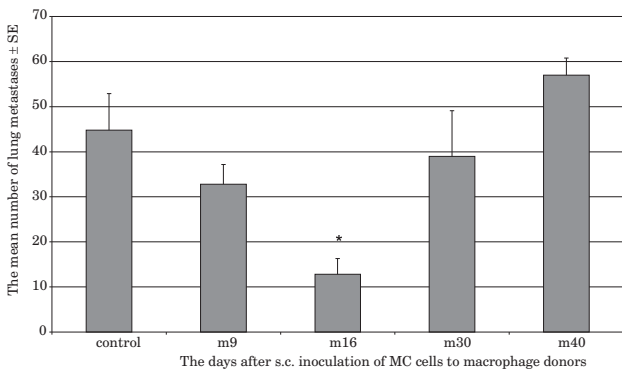


Fig. 2. The antimetastatic effect of adoptively transferred macrophages from various periods of concomitant tumor immunity. Animals in control group received only  $1 \times 10^6$  MC cells i.v. Adoptive transfer was performed by i.v. injection of  $8 \times 10^6$  macrophages following by the i.v. injection of  $1 \times 10^6$  MC cells. Indices: 9, 16, 30 and 40 refers to macrophages from mice carrying MC subcutaneously for 9, 16, 30 or 40 days.

\* $p \leq 0.05$  versus control

TABLE 2  
THE ANTIMETASTATIC EFFECT OF ADOPTIVELY TRANSFERRED MACROPHAGES FROM VARIOUS PERIODS OF CI ADDED TO CONCOMITANT IMMUNE SPLENOCYTES (SPL<sub>9</sub>)

Group	Adoptively transferred spleen cells*	The mean number of lung metastases ± SE
1	—	42.2 ± 1.8 <sup>#</sup>
2	spl <sub>9</sub>	12.0 ± 1.5
3	spl <sub>9</sub> + m <sub>9</sub>	10.6 ± 1.5
4	spl <sub>9</sub> + m <sub>16</sub>	4.8 ± 1.4 <sup>#</sup>
5	spl <sub>9</sub> + m <sub>30</sub>	30.0 ± 1.8 <sup>#</sup>
6	spl <sub>9</sub> + m <sub>40</sub>	45.5 ± 2.1 <sup>#</sup>

\* Animals in group 1 received only  $1 \times 10^6$  MC cells i.v. Adoptive transfer was performed by i.v. injection of  $8 \times 10^6$  concomitant immune splenocytes (spl<sub>9</sub>). Additionally to spl<sub>9</sub> groups 3, 4, 5 and 6 received  $8 \times 10^6$  macrophages. Indices: 9, 16, 30 and 40 refers to macrophages from mice carrying MC subcutaneously for 9, 16, 30 or 40 days. Adoptive transfer was followed up by the i.v. injection of  $1 \times 10^6$  MC cells the day after.

<sup>#</sup> $p \leq 0.01$  versus group 2

mals carrying MC for 30 or 40 days diminished anti-metastatic effect of spl<sub>9</sub> (Groups 5 and 6). Those results indicate that macrophages are responsible for the late period of CI weakening and suppression.

To investigate whether prostaglandins can mediate suppressive effect of macrophages from different periods of CI weakening on concomitant immune splenocytes, we used experiments with indomethacin. First we ruled out the possible direct antimetastatic effect of indomethacin (data not shown). Mice were adoptively transferred with concomitant immune splenocytes and macrophages from mice treated with indomethacin for 2 weeks before tumor injection and carrying MC for 16, 30 and 40 days (Table 3). Macrophages originating from the animals car-

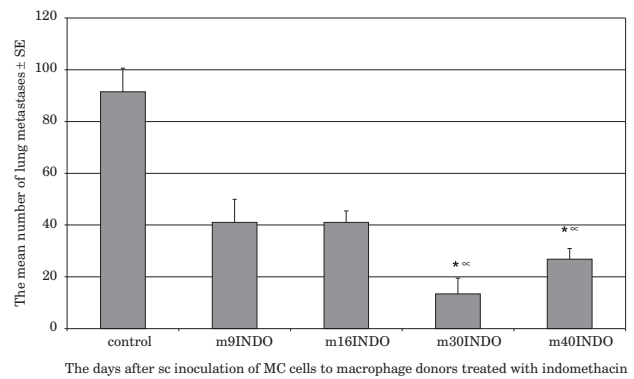


Fig. 3. The antimetastatic effect of adoptively transferred macrophages from various periods of CI; the macrophage donors were treated with indomethacin.

Animals in control group received only  $1 \times 10^6$  MC cells i.v. Adoptive transfer was performed by i.v. injection of  $8 \times 10^6$  macrophages from donors treated with indomethacin following by the i.v. injection of  $1 \times 10^6$  MC cells. Indices: 9, 16, 30 and 40 refers to macrophages from mice carrying MC subcutaneously for 9, 16, 30 or 40 days.

\* $p \leq 0.001$  versus control; ?  $p \leq 0.05$  versus m<sub>9</sub>INDO

TABLE 3  
THE ANTIMETASTATIC EFFECT OF ADOPTIVELY TRANSFERRED MACROPHAGES FROM VARIOUS PERIODS OF CI ADDED TO CONCOMITANT IMMUNE SPLENOCYTES (SPL<sub>9</sub>); THE MACROPHAGE DONORS WERE TREATED WITH INDOMETHACIN

Group	Adoptively transferred spleen cells*	The mean number of lung metastases ± SE
1	—	49.3 ± 2.3 <sup>#</sup>
2	spl <sub>9</sub>	11.8 ± 1.4
3	spl <sub>9</sub> + m <sub>16</sub> INDO	4.5 ± 1.6 <sup>#</sup>
4	spl <sub>9</sub> + m <sub>30</sub> INDO	5.7 ± 1.5 <sup>#</sup>
5	spl <sub>9</sub> + m <sub>40</sub> INDO	7.7 ± 1.4

\* Animals in group 1 received only  $1 \times 10^6$  MC cells i.v. Adoptive transfer was performed by i.v. injection of  $8 \times 10^6$  concomitant immune splenocytes (spl<sub>9</sub>). Additionally to spl<sub>9</sub> groups 3, 4 and 5 received  $8 \times 10^6$  macrophages from donors treated with indomethacin. Indices: 16, 30 and 40 refers to macrophages from mice carrying MC subcutaneously for 16, 30 or 40 days. Adoptive transfer was followed up by the i.v. injection of  $1 \times 10^6$  MC cells the day after.

<sup>#</sup> $p \leq 0.05$  versus group 2

rying MC for 30 or 40 days and treated with indomethacin lost their suppressive effect if being added to concomitant immunogenic splenocytes. Furthermore, antimetastatic effect of concomitant immunogenic splenocytes became more pronounced (Table 3).

To clarify the mechanism of macrophage suppressive effect; mice were adoptively transferred with  $8 \times 10^6$  macrophages from mice carrying subcutaneous MC for 9, 16, 30 and 40 days and treated with indomethacin for two weeks *a priori* the experiment. One day after adoptive transfer, animals were intravenously injected with

MC cells. Results are shown in Figure 3. The number of lung metastases after adoptive transfer of macrophages taken from the mice carrying subcutaneous MC for 30 and 40 days and treated with indomethacin decreased as compared to the control (Figure 3). These macrophages originating from the treated animals not only kept their antimetastatic activity, but also that effect was more pronounced as compared to the macrophages taken from the animals carrying MC for 9 and 16 days. These results suggest that inhibition of prostaglandine  $E_2$  synthesis by indomethacin restored antimetastatic effect of concomitant immune spleen cells.

## Discussion

Concomitant immunity is a phenomenon recorded in many tumor models, but its appearance time, development and weakening depend on tumor type and experimental model used<sup>19</sup>. Previously we analyzed the dynamic of CI in mammary carcinoma experimental model (both primary and secondary tumors grow s.c.). In that model CI attains maximal intensity about day 9 after injection of primary tumor and becomes weaker after 16 day<sup>20</sup>. In here present study, splenocytes from mice carrying MC for 30 or 40 days don't have any antimetastatic effect when being adoptively transferred (Figure 1). Furthermore, splenocytes with the best antimetastatic effect (spl<sub>9</sub>) lost their effect partially when being added to the splenocytes from mice carrying MC for 16 days and completely when being added to the splenocytes from mice carrying MC for 30 or 40 days (Table 1). That could be explained with the existence of immunosuppressive cells that appear in that time in the spleen and that have been previously shown to be macrophages<sup>15,21</sup>. To confirm that, we adoptively transferred macrophages from different period of CI. Since macrophages showed antimetastatic effect on day 16, we assumed that antimetastatic effect before that day was the act of lymphocytes as is in concordance with literature<sup>15</sup>, while later on the role was overtaken by macrophages (Figure 2). Moreover, the adoptive transfer of macrophages from animal carrying tumors for 30 and 40 days increased lung metastases number and this confirmed the role of macrophages in tumor progression<sup>22</sup>. We confirmed that splenocytes with the best antimetastatic effect (spl<sub>9</sub>) lost their influence when being added to the macrophages from mice carrying MC for 30 or 40 days (Table 2).

Many authors have reported that macrophage acts via secretion various cytokines such as prostaglandin  $E_2$ <sup>13,23</sup>. Results from the experiment using adoptive transfer of concomitant immunogenic splenocytes and macrophages from different stages of CI showed that certain macrophages have lost their suppressive effect (Figure 3). That includes macrophages that were taken 30 or 40 days following subcutaneous MC injection from the animals

treated with indomethacin for two weeks previously. Moreover, these macrophages even increased antitumor effect of concomitant immunogenic splenocytes. Our results indicated that prostaglandins are responsible for suppressive macrophage effect in a period of CI weakening. However, beside  $PGE_2$  the role of other factors in suppressive macrophage effect, such as nitric oxide, can't be excluded<sup>24,25</sup>.

The mechanism of macrophage suppressive effect in period of CI weakening could be explained in two ways: 1) in that time there are cytotoxic macrophages whose cytotoxicity is inhibited by suppressive macrophages, suggesting the existence of two different populations of macrophages; one that acts suppressive and the other that is being suppressed; and 2) it is possible that cytotoxic macrophages have lost their antitumor's effect 30 days following MC injection and consequently became suppressive cells, suggesting the existence of only one suppressive population of macrophages. Results shown in Table 3 indicate that there are two subpopulations of macrophages. Namely, better antimetastatic effect of cell suspension made of concomitant immunogenic splenocytes and macrophages taken on the day 30 and 40 from the animals treated with indomethacin could be explained by the inhibition of suppressive macrophages by indomethacin. In that way, cytotoxic macrophages that were suppressed by that time, could become active and intensify the effect of concomitant immunogenic splenocytes. Results shown in Figure 3 are in consistence with that explanation. If macrophages that were taken from animals carrying MC for 30 or 40 days, and treated with indomethacin, are being adoptively transferred they have strong antitumor effect, what differs from the macrophages taken from untreated animals under same other conditions (Figure 2). That phenomenon suggests that cytotoxic macrophages act freely without inhibition of suppressive macrophages.

Important to mention is the fact that macrophages whose antimetastatic effect we studied, originated from spleen, and we didn't investigated the role of alveolar macrophages in metastasing process. Also, it has to be kept on mind that macrophages are not the only source of prostaglandins in tumor host and that various tumor types secrete great amounts of  $PGE_2$ <sup>26</sup>. Since, tumor cells can be the source of prostaglandins (8), the indomethacin effect on prostaglandins originating from tumor must be also taken into consideration.

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## DJELOVANJE INDOMETACINA NA ANTIMETASTASKI UČINAK MAKROFAGA: MODEL POPRATNE IMUNOSTI

### SAŽETAK

Uloga makrofaga kao imunološkog antitumorskog čimbenika ili poticatelja tumorskog rasta još je uvijek puna nepoznanica. Istražili smo funkciju makrofaga u modelu pridružene imunosti koja se opisuje kao pojava odbacivanja sekundarnog tumora za vrijeme rasta primarnog tumora. Naime, pokazano je kako se za vrijeme slabljenja pridružene imunosti mogu pojaviti tumorske metastaze. Upotrijebili smo arteficialne plućne metastaze karcinoma dojke kako bismo istražili utjecaj makrofaga iz različitih faza pridružene imunosti na razvoj mišjih metastaza. Rezultati su pokazali kako su makrofazi odgovorni za supresiju i kasnu fazu slabljenja pridružene imunosti. Kako bismo istražili jesu li prostaglandini odgovorni za supresivne učinke makrofaga iskoristili smo pokuse s indometacinom. Dokazali smo kako inhibicijom sinteze prostaglandina, indometacin oporavlja antimetastatski učinak makrofaga pridružene imunosti.